



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Tumor necrosis factor alpha-deficient, but not interleukin-6-deficient, mice resist peripheral infection with scrapie

Citation for published version:

Mabbott, N, Williams, A, Farquhar, C, Pasparakis, M, Kollias, G & Bruce, M 2000, 'Tumor necrosis factor alpha-deficient, but not interleukin-6-deficient, mice resist peripheral infection with scrapie' *Journal of Virology*, vol 74, no. 7, pp. 3338-44.

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Publisher final version (usually the publisher pdf)

Published In:

Journal of Virology

Publisher Rights Statement:

Copyright © 2000, American Society for Microbiology

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



Tumor Necrosis Factor Alpha-Deficient, but Not Interleukin-6-Deficient, Mice Resist Peripheral Infection with Scrapie

NEIL A. MABBOTT,^{1*} ALUN WILLIAMS,^{1†} CHRISTINE F. FARQUHAR,¹ MANOLIS PASPARAKIS,²
GIORGOS KOLLIAS,² AND MOIRA E. BRUCE¹

*Neuropathogenesis Unit, Institute for Animal Health, Edinburgh EH9 3JF, Scotland, United Kingdom,¹
and Hellenic Pasteur Institute, 115 21 Athens, Greece²*

Received 21 October 1999/Accepted 4 January 2000

In most peripheral infections of rodents and sheep with scrapie, infectivity is found first in lymphoid tissues and later in the central nervous system (CNS). Cells within the germinal centers (GCs) of the spleen and lymph nodes are important sites of extraneural replication, from which infection is likely to spread to the CNS along peripheral nerves. Here, using immunodeficient mice, we investigate the identity of the cells in the spleen that are important for disease propagation. Despite possessing functional T and B lymphocytes, tumor necrosis factor alpha-deficient ($\text{TNF-}\alpha^{-/-}$) mice lack GCs and follicular dendritic cell (FDC) networks in lymphoid tissues. In contrast, lymphoid tissues of interleukin-6-deficient ($\text{IL-6}^{-/-}$) mice possess FDC networks but have impaired GCs. When the CNSs of $\text{TNF-}\alpha^{-/-}$, $\text{IL-6}^{-/-}$, and wild-type mice were directly challenged with the ME7 scrapie strain, 100% of the mice were susceptible, developing disease after closely similar incubation periods. However, when challenged peripherally (intraperitoneally), most $\text{TNF-}\alpha^{-/-}$ mice failed to develop scrapie up to 503 days postinjection. All wild-type and $\text{IL-6}^{-/-}$ mice succumbed to disease approximately 300 days after the peripheral challenge. High levels of scrapie infection and the disease-specific isomer of the prion protein, PrP^{Sc} , were detectable in spleens from challenged wild-type and $\text{IL-6}^{-/-}$ mice but not from $\text{TNF-}\alpha^{-/-}$ mice. Histopathological analysis of spleen tissue demonstrated heavy PrP accumulations in direct association with FDCs in challenged wild-type and $\text{IL-6}^{-/-}$ mice. No PrP^{Sc} accumulation was detected in spleens from $\text{TNF-}\alpha^{-/-}$ mice. We conclude that, for the ME7 scrapie strain, mature FDCs are critical for replication in lymphoid tissues and that in their absence, neuroinvasion following peripheral challenge is impaired.

The transmissible spongiform encephalopathies (TSEs), or prion diseases, comprise a closely related group of neurodegenerative disorders that include Creutzfeldt-Jakob disease (CJD) and kuru in humans, scrapie in sheep and goats, transmissible mink encephalopathy, and bovine spongiform encephalopathy in cattle. The TSEs are characterized by the deposition within the brain of PrP^{Sc} (4), an abnormal, detergent-insoluble, relatively protease-resistant isoform of a normal host cellular protein, PrP^{C} (37). While the precise nature of the agent is still the subject of controversy (11), PrP^{Sc} copurifies with infectivity (40) and is considered to be a major component of the infectious agent. Furthermore, mice deficient in the PrP gene ($\text{PrP}^{-/-}$ mice) do not develop TSE disease (7), demonstrating that host cells must express PrP in order to propagate infection.

Natural TSE infections are most often acquired by peripheral routes of infection. Disease may be established through the skin (by scarification), orally (through foodstuffs or cannibalism), or, in some instances of CJD in humans, iatrogenically through transplantation of CJD-contaminated tissues or pituitary-derived hormones. In the United Kingdom, the consumption of beef products contaminated with bovine spongiform encephalopathy is the most likely cause of variant CJD (vCJD) in humans (6).

Following peripheral infection of sheep or rodents with

scrapie, high titers of infectivity rapidly accumulate in the spleen. Lymphoid tissues play an important role in pathogenesis, as genetic asplenia or splenectomy of mice, prior to or shortly after a peripheral scrapie challenge, significantly extends the incubation period of the disease (15). From the lymphoid tissues, infectivity is considered to gain access to the central nervous system (CNS) via its spread along peripheral nerves (1, 35). Although it has been suggested that TSE infection may enter the CNS by hematogenous spread (22), there is no firm evidence to support this. Once scrapie infection enters the CNS, the neurodegeneration it causes is irreversible and death is inevitable.

Thymectomy does not affect the incubation period of the disease following peripheral challenge (15) and neither does ionizing irradiation (16), implying that scrapie pathogenesis depends on radioresistant and mitotically inactive cells. Ionizing irradiation eliminates T lymphocytes, their precursors, and actively dividing B lymphocytes as potential sites of scrapie replication. Follicular dendritic cells (FDCs) fulfil the above criteria and also decorate strongly with anti- PrP antibodies, even in uninfected mice (34, 43). In severe combined immunodeficient (SCID) mice, the absence of mature B and T lymphocytes prevents the maturation of FDCs (20). These mice resist peripheral infection with the scrapie strain ME7 (13, 45) or the human TSE strain Fukuoka-2 (21) and fail to accumulate infectivity and PrP^{Sc} in their spleens. Maturation of FDCs and germinal centers (GCs), along with susceptibility to scrapie, can be induced by grafting SCID mice with bone marrow (13). As FDC networks do not develop in the absence of lymphocytes (20) and both FDCs (5, 34, 43) and lymphocytes (31) appear to express PrP^{C} , further experiments using mice that expressed and did not express PrP^{C} in their FDCs and lympho-

* Corresponding author. Mailing address: Institute for Animal Health, Neuropathogenesis Unit, Ouston Building, West Mains Rd., Edinburgh EH9 3JF, Scotland, United Kingdom. Phone: 44 131 667 5204. Fax: 44 131 668 3872. E-mail: neil.mabbott@bbsrc.ac.uk.

† Present address: Department of Veterinary Pathology, Glasgow University Veterinary School, Glasgow G61 1QH, Scotland, United Kingdom.

cytes have been undertaken to determine the role of these cells in scrapie pathogenesis (5).

Signalling between FDCs and lymphocytes through cytokines, including tumor necrosis factor alpha (TNF- α) and interleukin-6 (IL-6), plays an important role in the organization of the GC. Mice deficient in TNF- α lack splenic primary B-cell follicles, FDC networks, and GCs (38). Despite the absence of GC structure, B lymphocytes are still able to respond to antigen stimulation and immunoglobulin class-switching can still occur. The effects of TNF- α on GC architecture are mediated via signalling through TNF receptor 1 (TNF-R1) expressed on FDCs and/or its precursor (46). IL-6 production by FDCs has also been shown to be important for maintaining GC reactions. In the lymphoid tissues of mice deficient for IL-6, FDC complexes are able to form but GC development is diminished (26). To determine if FDCs, GCs, and lymphocytes are required for susceptibility to a peripherally routed infection, scrapie pathogenesis was studied in mice which lack FDCs and GCs but possess functional lymphocytes (TNF- α -deficient mice) and in mice in which FDC networks are present but GC development is impaired (IL-6-deficient mice).

MATERIALS AND METHODS

Mice. TNF- α knockout (TNF- $\alpha^{-/-}$ [38]) and IL-6 knockout (IL-6 $^{-/-}$ [25]) mice were bred on a mixed 129/Sv \times C57BL/6 background. Wild-type (control) mice also shared the 129/Sv \times C57BL/6 background.

Scrapie inoculation. Mice were injected intracerebrally (i.c.) or intraperitoneally (i.p.) with 20 μ l of a 1.0% (wt/vol) dilution of unspun brain homogenate from C57BL mice terminally affected with scrapie strain ME7. Following challenge, animals were coded and scored weekly to determine the clinical end point and incubation period of neurological disease, according to previously established criteria (14). Scrapie diagnosis was confirmed by histopathological assessment of vacuolation and PrP immunostaining in the brain. Where indicated, some mice were sacrificed 5 and 10 weeks postchallenge and spleens were taken for further analysis. For bioassay of scrapie infectivity, individual spleen halves were prepared as 10% homogenates in physiological saline and injected i.c. into C57BL assay mice. The scrapie titer in each spleen was determined from the mean incubation period in the assay mice, by reference to established dose-incubation period response curves for scrapie-infected spleen tissue.

Immunoblot detection of PrP^{Sc}. Tissues were prepared by a modification of a method previously described (10). Briefly, frozen tissues were weighed and pulverized in precooled Potter homogenizers and then homogenized in 0.2 M potassium chloride (2 ml) with 20 μ l of each of the protease inhibitors, 100 mM phenylmethylsulfonyl fluoride (PMSF) and 100 mM *N*-ethylmaleimide (NEM), both in propan-1-ol. Suspensions were then centrifuged at 500 \times g for 10 min at 4°C. The supernatants were decanted and centrifuged for 30 min at 100,000 \times g at 4°C. The resultant pellets were resuspended in 2 ml of 100 mM Tris-HCl at pH 7.4, and the suspension was divided into two equal parts. To one fraction, 20 μ l of 20-mg/ml proteinase K (Sigma, Poole, United Kingdom) was added, and the contents were incubated at 37°C in an orbital shaker for 60 min; the other fraction was held at 4°C with 20 μ l each of PMSF and NEM (100 mM). Subsequently, 1 ml of Sarkosyl (2%, wt/vol), 20 μ l each of PMSF and NEM, and 2 μ l of 2-mercaptoethanol were added to all tubes, which were incubated at 37°C for a further 60 min. The contents of each tube were then layered onto a cushion of 20% sucrose in 50 mM Tris-HCl (pH 7.4) and centrifuged at 100,000 \times g for 2 h at 4°C. Pellets were drained and stored at -70°C before further analysis.

Samples were then electrophoresed through sodium dodecyl sulfate-12% polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hemel Hempstead, United Kingdom) by semidry blotting. Membranes were blocked with 2% bovine serum albumin in 0.1 M Tris-HCl (pH 7.6) and probed with the PrP-specific rabbit polyclonal antiserum 1B3 (12). Following counter-staining with alkaline phosphatase-conjugated goat anti-rabbit antiserum (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.), bound alkaline phosphatase activity was detected with SigmaFast nitroblue tetrazolium-BCIP (5-bromo-4-chloro-3-indolylphosphate) solution (Sigma).

Immunohistochemical analysis. Spleen halves were snap-frozen and maintained at the temperature of liquid nitrogen. Serial 6- μ m-thick sections were cut on a cryostat, air dried, and fixed in acetone for 10 min. FDCs were visualized by staining first with FDC-M1 monoclonal antiserum (28) and subsequently with biotinylated murine anti-rat serum. To detect PrP, sections were stained first with 1B3 polyclonal antiserum and subsequently with biotinylated goat anti-rabbit serum (Jackson ImmunoResearch Laboratories). To visualize GC B cells, sections were stained with biotinylated peanut agglutinin (Vector Laboratories, Peterborough, United Kingdom). Immunolabeling was carried out using alkaline phosphatase coupled to the avidin-biotin complex (Vector Laboratories). Vector Red (Vector Laboratories) was used as a substrate.

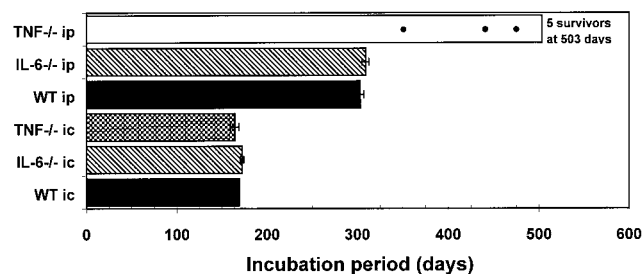


FIG. 1. Incubation period of disease following i.c. or i.p. challenge of wild-type (WT), IL-6 $^{-/-}$, and TNF- $\alpha^{-/-}$ (TNF- $\alpha^{-/-}$) mice with scrapie strain ME7. Each bar represents the mean \pm the standard error for 5 to 14 mice. While most i.p. challenged TNF- $\alpha^{-/-}$ mice remained free of the clinical signs of scrapie 503 days postinfection, three mice did succumb to disease (dots).

For the detection of PrP in brain tissue, brains were fixed in periodate-lysine-paraformaldehyde and embedded in paraffin wax. Sections (thickness, 6 μ m) were deparaffinized, and pretreated to enhance PrP immunostaining by hydrated autoclaving (15 min, 121°C, hydration), and subsequent immersion in formic acid (98%) for 5 min (34). Sections were then stained with the PrP-specific antiserum 1B3 or normal rabbit serum as a control and immunocytochemical labeling was carried out using the peroxidase-antiperoxidase technique with diaminobenzidine as a substrate. Sections were counterstained with hematoxylin to distinguish cell nuclei.

Immunohistoblot analysis of PrP^{Sc}. Spleen halves were snap-frozen and maintained at the temperature of liquid nitrogen. Serial 10- μ m-thick sections were cut on a cryostat, applied directly to polyvinylidene difluoride membranes, and thoroughly air dried. For the detection of total PrP (PrP^C and PrP^{Sc}) and PrP^{Sc} alone, membranes were rehydrated and treated in the absence and presence (respectively) of 20 μ g of proteinase K per ml for 60 min at 37°C, as previously described (44). Following processing, membranes were probed with PrP-specific polyclonal antiserum 1B3 and counter-stained with alkaline phosphatase-conjugated goat anti-rabbit antiserum and bound alkaline phosphatase activity was detected with SigmaFast nitroblue tetrazolium-BCIP solution.

RESULTS

Susceptibility of TNF- $\alpha^{-/-}$ and IL-6 $^{-/-}$ mice to scrapie infection. When mice were challenged i.c. with scrapie strain ME7, no significant differences between wild-type, TNF- $\alpha^{-/-}$, and IL-6 $^{-/-}$ mice in the onset of clinical signs or incubation period of disease were observed (Fig. 1). All i.c. infected mice succumbed to disease approximately 170 days postchallenge. Histopathologic analysis of brain tissue from terminal i.c. infected wild-type, IL-6 $^{-/-}$, and TNF- $\alpha^{-/-}$ mice showed the characteristic spongiform pathology and PrP^{Sc} accumulation associated with ME7. Thus, if scrapie was delivered directly to the CNS, scrapie pathogenesis in the brain proceeded without any detectable influence of the immune status of the host.

When mice were challenged with scrapie peripherally via the i.p. route, no significant difference was observed between wild-type and IL-6 $^{-/-}$ mice in the incidence and incubation period of disease. With both of these mouse strains, all animals succumbed to infection following mean incubation periods of 303 \pm 4 days (wild-type mice; n = 8) and 309 \pm 4 days (IL-6 $^{-/-}$ mice; n = 14) (Fig. 1). Furthermore, spongiform pathology, gliosis, and PrP^{Sc} accumulation typical of an i.p. infection with scrapie strain ME7 were detected in the brains of wild-type and IL-6 $^{-/-}$ i.p. challenged mice (Fig. 2a and b, respectively). In contrast, following i.p. challenge of TNF- $\alpha^{-/-}$ mice with scrapie, five of eight mice remained free of signs of disease up to 503 days postinfection, at which time the experiment was terminated (Fig. 1). No evidence of spongiform change or PrP^{Sc} accumulation was detected in the brains of any surviving i.p. challenged TNF- $\alpha^{-/-}$ mice (Fig. 2c). However, three of eight TNF- $\alpha^{-/-}$ mice did succumb to an i.p. challenge with scrapie. In these, disease developed after individual incubation periods of 350, 441, and 475 days. These incubation periods

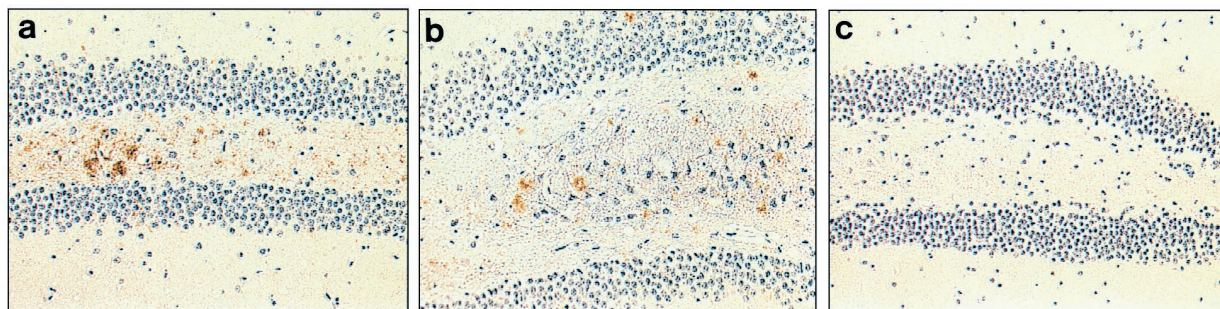


FIG. 2. Immunocytochemical analysis of brain tissue from i.p. challenged, terminally scrapie-affected wild-type (a) and IL-6^{-/-} (b) mice detected large PrP accumulations (brown) in the hippocampus. In contrast, no PrP accumulations were detected in tissue derived from i.p. challenged TNF-α^{-/-} mice that remained free of the signs of scrapie 503 days postinfection (c). All sections were counterstained with hematoxylin (blue). Magnification, ×200.

were beyond the range seen in i.p. infected wild-type and IL-6^{-/-} mice (289 to 325 and 280 to 325 days, respectively).

Scrapie infectivity and PrP^{Sc} accumulation in the spleen. High levels of scrapie infectivity were detected in spleens from wild-type mice collected 35, 70, and 310 days postchallenge, by which time the mice were terminally affected with scrapie (Table 1). Interestingly, no infectivity has been detected so far in one of four spleens collected 35 days postchallenge, suggesting a titer less than 2.5-log-unit i.c. 50% infective doses (ID₅₀)/g. However, by 70 days postchallenge, high levels of infection were detected in all four spleens assayed. In contrast, scrapie infectivity has so far been undetectable in all of the spleens taken from scrapie-challenged TNF-α^{-/-} mice 35, 70, and 503 days postchallenge (Table 1). While most indicator mice injected with tissue from infected-wild-type mice succumbed to TSE disease between 175 and 210 days postchallenge, all those injected with tissue from challenged TNF-α^{-/-} mice remained free of TSE disease up to at least 400 days postchallenge. This represents an infectious titer, if present, below 2.0-log-unit i.c. ID₅₀/g (the limit of detection of the assay), at least 1,000-fold less than the titer measured in spleens from wild-type mice. High levels of scrapie infectivity were detected in all spleens collected from IL-6^{-/-} mice 35 and 70 days postchallenge (Table 1).

Immunoblot analysis of tissue from i.p. challenged, terminally scrapie-affected wild-type and IL-6^{-/-} mice detected large accumulations of detergent-insoluble, proteinase K-resistant PrP^{Sc} in the spleen (Fig. 3a). In contrast, no PrP^{Sc} was

detected in spleens derived from i.p. challenged TNF-α^{-/-} mice, which remained free of the signs of scrapie 503 days postinfection (Fig. 3a), or from any of the three TNF-α^{-/-} mice that succumbed to disease (Fig. 3b).

Large FDC networks and peanut agglutinin-positive GCs were detected by immunohistochemistry in the spleens of wild-type mice (Fig. 4a and d, respectively). In spleens from IL-6^{-/-} mice, FDC networks were detected but GCs were impaired and considerably smaller than those from wild-type mice (Fig. 4b and e, respectively). Neither mature FDCs nor GCs were detected in tissues from TNF-α^{-/-} mice (Fig. 4c and f, respectively). Analysis of adjacent sections of spleen from both wild-type and IL-6^{-/-} mice demonstrated large PrP accumulations (Fig. 4j and k, respectively) in direct association with FDCs (Fig. 4g and h, respectively) in tissue taken 10 weeks post-peripheral challenge with scrapie. No FDCs or PrP accumulations were detected immunocytochemically in the spleens of TNF-α^{-/-} mice (Fig. 4i and l, respectively). Further analysis of adjacent cryostat sections by immunohistoblotting demonstrated that in spleens from scrapie-challenged wild-type mice, large proteinase K-resistant PrP^{Sc} accumulations (Fig. 5g) occurred in direct association with FDCs (Fig. 5a). No PrP^{Sc} accumulations or FDCs were detected in spleens from scrapie-challenged TNF-α^{-/-} mice (Fig. 5b and h, respectively). In spleens from uninfected wild-type mice, only the proteinase K-sensitive, cellular isomer of the prion protein, PrP^C, was detected in association with FDCs (Fig. 5f, i, and c, respectively). Taken together, these observations demonstrate that following peripheral challenge with scrapie strain ME7, PrP^{Sc} accumulates in the spleen in direct association with FDCs. Interestingly, no PrP^C was detectable by immunoblot analysis in spleens derived from TNF-α^{-/-} mice (Fig. 3), although low levels were detected in spleens from uninfected wild-type animals by immunoblot analysis (data not shown) and immunohistoblot analysis (Fig. 5f). This difference is most likely due to the lack of FDCs expressing high levels of PrP^C in the spleens of TNF-α^{-/-} mice.

DISCUSSION

The susceptibility of TNF-α^{-/-} mice to peripheral challenge with scrapie in this study was greatly reduced in comparison with that of immunocompetent wild-type mice. We also demonstrated that TNF-α^{-/-} mice, unlike wild-type mice, failed to accumulate scrapie infectivity in their spleens. However, following peripheral challenge of IL-6^{-/-} mice with scrapie, no significant differences in incubation period or incidence of disease were observed between mutant and wild-type mice. Likewise, high levels of scrapie infectivity were detected in the

TABLE 1. Scrapie infectivity titers in spleens from wild-type, IL-6^{-/-}, and TNF-α^{-/-} mice following i.p. challenge with scrapie strain ME7

Mouse strain	Levels of scrapie infection in individual mouse spleens (log i.c. ID ₅₀ /g) at day postchallenge ^a :			
	35	70	310 ^b	503 ^c
Wild type	4.4, 4.9, 4.9, UD ^d	4.7, 4.8, 5.7, 5.8	5.0, 5.7	— ^e
IL-6 ^{-/-}	4.8, 5.0	5.0, 5.7	—	—
TNF-α ^{-/-}	UD, UD	UD, UD	—	UD, UD

^a At the indicated times postchallenge, scrapie infectivity titers in individual spleens were determined by i.c. injection of 20 μl of a 10% (wt/vol) spleen homogenate into groups of 5 to 12 C57BL mice.

^b Time at which wild-type mice were clinically affected with scrapie.

^c Brains from the individual TNF-α^{-/-} mice assayed were free of the signs of scrapie at this time postchallenge.

^d UD, scrapie infection undetectable. All recipient mice remained free of the signs of TSE disease at least 400 days postinjection, indicating a titer of <2.0 log-unit i.c. ID₅₀/g.

^e —, not done.

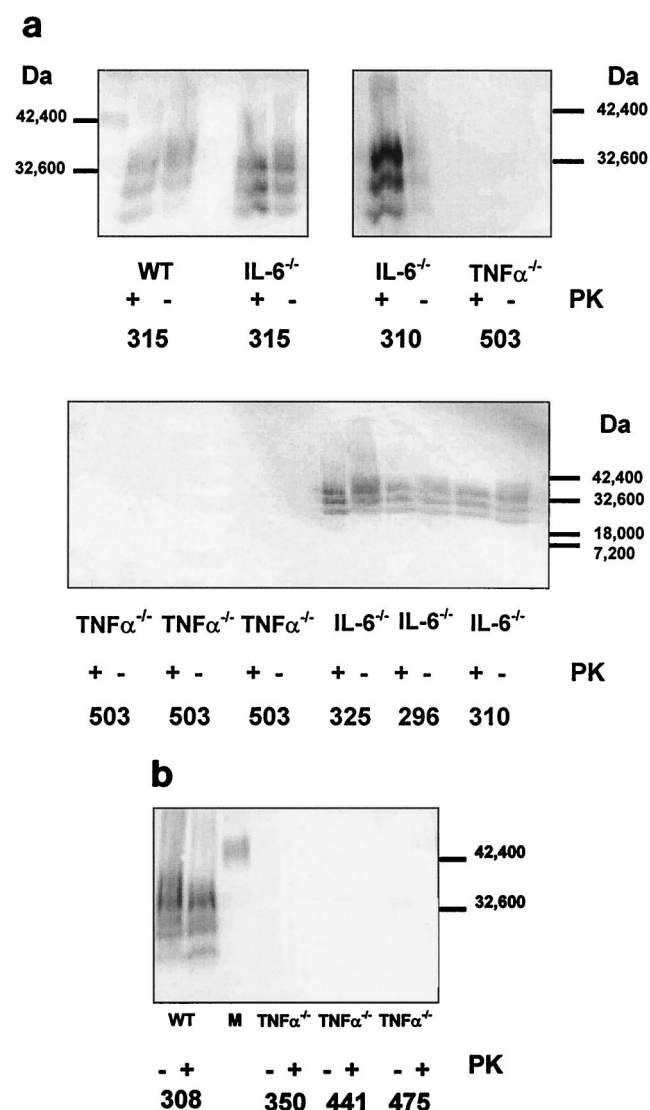


FIG. 3. Immunoblot analysis of spleen tissue from wild-type (WT), TNF- $\alpha^{-/-}$, and IL-6 $^{-/-}$ mice following intraperitoneal challenge with the ME7 scrapie strain. Samples were treated in the presence (+) or absence (-) of proteinase K (PK) before electrophoresis. (a) Large PK-resistant accumulations of PrP^{Sc} were detected in spleens derived from clinically scrapie-affected WT and IL-6 $^{-/-}$ mice. No PrP^{Sc} accumulations were detected in spleens derived from scrapie-challenged TNF- $\alpha^{-/-}$ mice that remained free of disease 503 days postinfection. (b) Large PK-resistant accumulations of PrP^{Sc} were detected in spleens derived from clinically scrapie-affected WT mice but not in spleens derived from clinically affected TNF- $\alpha^{-/-}$ mice. Values given below lanes are the individual incubation periods of disease or times postchallenge at which the spleens were harvested. Lane M, molecular size markers.

spleens of challenged IL-6 $^{-/-}$ mice. The disease-specific isoform of the prion protein, PrP^{Sc}, is associated with high levels of scrapie infectivity (40) and provides a reliable indicator for the presence of infection in this model (10). Large PrP^{Sc} accumulations were detected in the spleens of scrapie-challenged wild-type and IL-6 $^{-/-}$ mice but not in the spleens of TNF- $\alpha^{-/-}$ mice. This was true even for the few TNF- $\alpha^{-/-}$ mice that developed disease after protracted incubation periods.

Overexpression of TNF- α has been implicated as a key pathogenic mediator in several human inflammatory, infectious, and autoimmune CNS disorders (39), including bacterial

meningitis (30), multiple sclerosis (42), cerebral malaria (17), and Alzheimer's disease (36). In rodents, neutralization of TNF- α prevents the formation of inflammatory lesions and demyelination caused by experimental autoimmune encephalomyelitis (24). Induction of IL-1 α , IL-1 β , IL-6, and TNF- α synthesis has been reported to occur in the brains of mice showing clinical signs of scrapie (47), suggesting that cytokines may be significant factors in determining the pathogenesis of neurodegeneration in scrapie. Similarly, expression of TNF- α and IL-1 α is increased in the brains of mice infected experimentally with the Fujisaki strain of human TSE (27). Here, when the CNSs of TNF- $\alpha^{-/-}$ and IL-6 $^{-/-}$ mice were infected directly with scrapie, no significant differences were observed between mutant and wild-type mice in either the onset of clinical disease or CNS pathology. Although cytokine production by activated glia precedes the apoptotic loss of hippocampal neurons in brains from scrapie-infected mice (47), the experiments presented here suggest that TNF- α and IL-6 alone do not play a critical role in the development of pathology in the CNS. Therefore, the apparent resistance of TNF- $\alpha^{-/-}$ mice to i.p. challenge with scrapie could not be attributed to a role of TNF- α in the development of pathology in the CNS. Our experiments strongly suggest that the effects of TNF- α operate at a peripheral stage, prior to neuroinvasion.

How scrapie infectivity is delivered to the lymphoid tissues is not known. As membrane lymphotoxin, instead of TNF- α , regulates the migration of dendritic cells in the spleen (48), it is unlikely that the effects of TNF- α deficiency on scrapie pathogenesis are due to impaired cell trafficking from the site of scrapie challenge to the spleen. Gene deletion experiments with mice have shown that signalling by both TNF- α and lymphotoxin α/β is required for FDC development (33, 38). The subsequent maintenance of FDCs in a differentiated state requires the continual stimulation of FDCs by B lymphocytes through lymphotoxin α/β and TNF- α (32). Hence, lymphoid tissues from TNF- $\alpha^{-/-}$ mice lack FDC networks and GCs but possess functional lymphocytes (38). In contrast, recent work suggests that IL-6 production by FDCs may act directly on GC B lymphocytes via IL-6-sensitive transcription factors and may also amplify local C3 synthesis by tingible body macrophages (25). This leads to impaired GC formation and antibody responses, but mice deficient in the production of IL-6 are able to form FDC complexes. Our experiments suggest that deficiencies in GCs alone do not affect scrapie pathogenesis, as peripherally challenged IL-6 $^{-/-}$ mice developed disease at the same time as wild-type mice and had high levels of scrapie infectivity and large PrP^{Sc} accumulations in their spleens. It is also unlikely that T and B lymphocytes are directly involved in disease pathogenesis, as these cells are present and functional in lymphoid tissues of TNF- $\alpha^{-/-}$ mice (38), which did not develop disease or accumulate scrapie infectivity or PrP^{Sc} in the spleen. Immunohistopathology demonstrated that the large PrP^{Sc} accumulations in the spleens of wild-type and IL-6 $^{-/-}$ mice were directly associated with FDCs. FDCs were completely absent in spleens from challenged TNF- $\alpha^{-/-}$ mice. We therefore conclude that scrapie accumulation in the spleen depends on the presence of mature FDCs. These findings are consistent with recent experiments that used mice that expressed and mice that did not express PrP^C in their FDCs and lymphocytes. In these experiments, replication of scrapie strain ME7 in the spleen was also dependent on PrP^C-expressing FDCs (5). From the spleen, infectivity is most likely to spread to the CNS along peripheral nerves (1, 34), as noradrenergic and peptidergic nerve fibers are present in both primary and secondary lymphoid organs among cells of the lymphoid follicles (2).

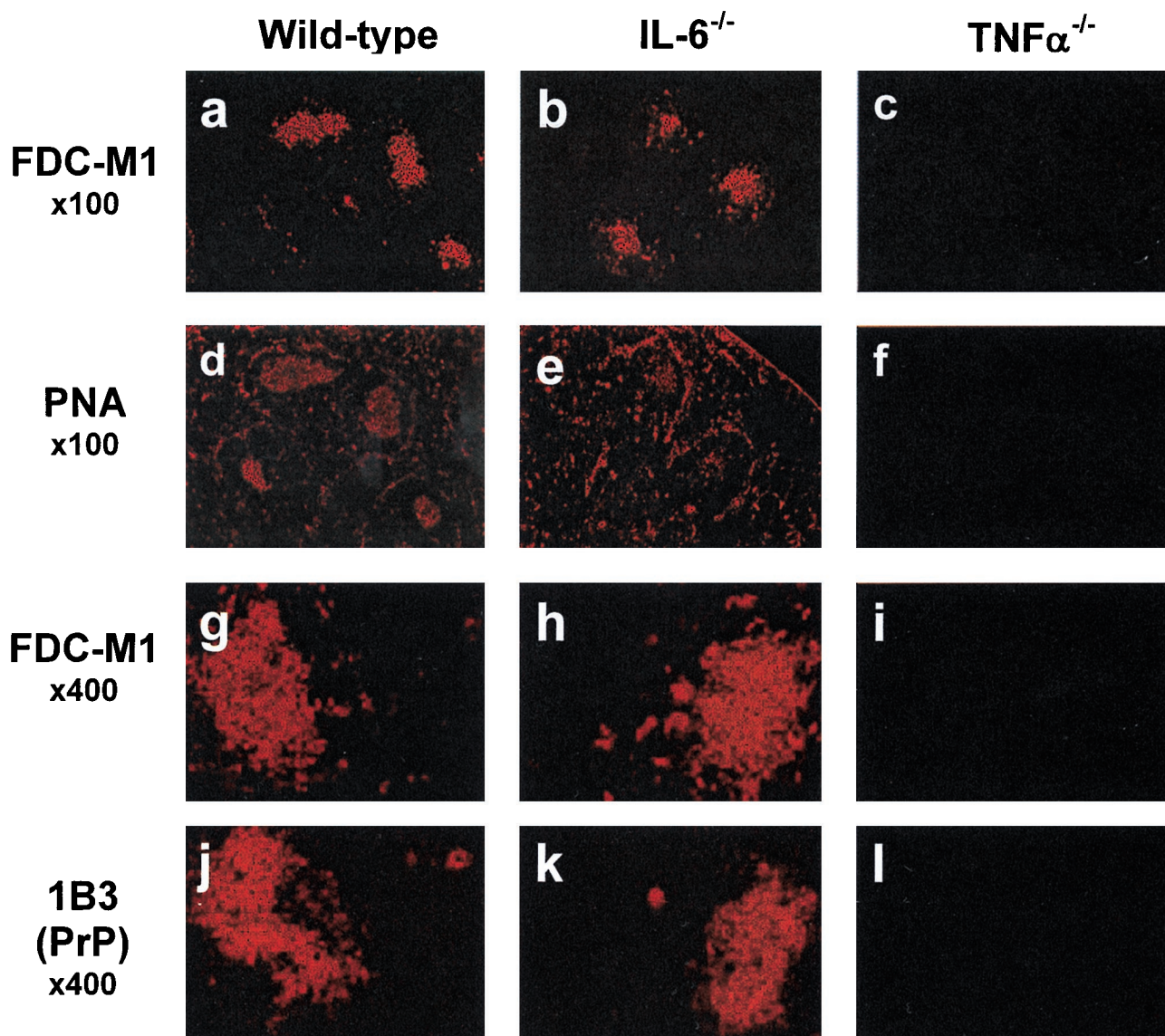


FIG. 4. Immunocytochemical analysis of GC structure and PrP accumulation in spleen tissue from ME7-challenged mice. (a to c) Sections from wild-type (a), IL-6^{-/-} (b), and TNF- α ^{-/-} (c) mice were stained with FDC-M1 antiserum to detect FDCs (red). (d to f) Adjacent sections from wild-type (d), IL-6^{-/-} (e), and TNF- α ^{-/-} (f) mice were stained with peanut agglutinin to detect GCs (red). (g to i) Sections from wild-type (g), IL-6^{-/-} (h), and TNF- α ^{-/-} (i) mice were stained with FDC-M1 (red). (j to l) Adjacent sections from wild-type (j), IL-6^{-/-} (k), and TNF- α ^{-/-} (l) mice were stained with the PrP-specific antiserum 1B3 (red). Magnification, $\times 100$ (a to f) and $\times 400$ (g to l).

Interestingly, three TNF- α ^{-/-} mice did succumb to scrapie following peripheral challenge, although with significantly longer incubation periods than challenged wild-type and IL-6^{-/-} mice. No PrP^{Sc} was detected in the spleens of any clinically scrapie-positive TNF- α ^{-/-} mice, suggesting that due to a lack of FDCs, spleen tissue is not involved in pathogenesis. Similarly, while SCID mice resist peripheral challenge with moderate doses of scrapie strain ME7, high doses produce CNS disease despite only trace levels of infection in the spleen (13). How scrapie infection had spread to the CNS in both of these instances is not known. Klein and colleagues (22) suggested that peripheral blood lymphocytes may carry infection from the periphery to the CNS. However, subsequent experiments have failed to detect infectivity in peripheral blood lymphocytes, despite high levels of infectivity in the spleen in this model (41). The most likely explanation is that in the absence

of a functional immune system, neurological disease follows direct uptake of infection by nerve endings in the periphery and spreads to the CNS along peripheral nerves (1).

The pathogenesis of scrapie strain ME7 following peripheral challenge appears to differ significantly from that of the scrapie isolate RML studied elsewhere; in that study infection accumulated in the spleen in the absence of PrP^c expression on FDCs (3). Further experiments using the RML scrapie isolate implicated B lymphocytes in disease pathogenesis, as mice deficient in B lymphocytes failed to accumulate infectivity in their spleens and were refractory to disease (22). However, as outlined above, mice deficient in B lymphocytes are also indirectly deficient in FDCs, as lymphocytes provide important signals for their maturation and maintenance (20, 32, 33). In order to distinguish between these two cell populations in the pathogenesis of the RML scrapie isolate, TNF-R1^{-/-} mice

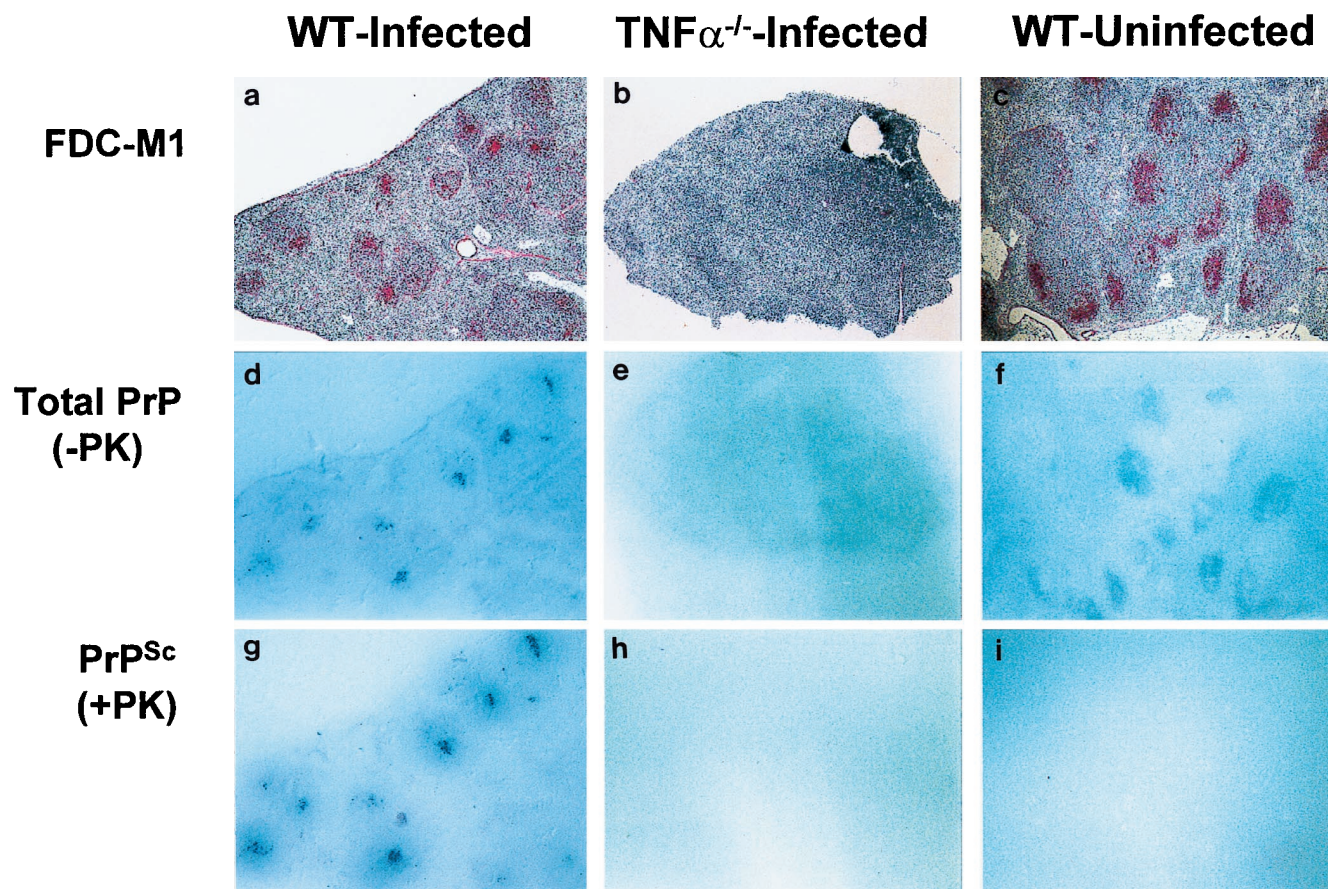


FIG. 5. Immunohistoblot analysis of PrP accumulation in the spleen. (a to c) Sections from scrapie-challenged wild-type (WT) (a), scrapie-challenged $\text{TNF-}\alpha^{-/-}$ (b), and uninfected wild-type (c) mice were stained with FDC-M1 antiserum to detect FDCs (red). (d to i) Histoblots were prepared from adjacent sections for specific staining of total PrP-PrP^c and PrP^{Sc}. (d to f) Immunodetection of total PrP-PrP^c in samples from scrapie-challenged wild-type (d), scrapie-challenged $\text{TNF-}\alpha^{-/-}$ (e), and uninfected wild-type (f) mice without prior treatment with proteinase K (-PK). (g to i) Immunodetection of PrP^{Sc} in samples from scrapie-challenged wild-type (g), scrapie-challenged $\text{TNF-}\alpha^{-/-}$ (h), and uninfected wild-type (i) mice with prior treatment with proteinase K (+PK). Following treatment, histoblots were stained with the PrP-specific antiserum 1B3. Magnification, $\times 40$.

were used. Like $\text{TNF-}\alpha^{-/-}$ mice, these mice lack mature FDCs but do possess functional lymphocytes (29, 33). Interestingly, $\text{TNF-R1}^{-/-}$ mice were as susceptible to peripheral challenge with the RML scrapie isolate as wild-type mice (22), implying that in the presence of PrP-expressing lymphocytes, FDCs are not critical for the pathogenesis of RML. In a third study, PrP expression on B lymphocytes was not critical for RML pathogenesis (23) in the presence of PrP-expressing FDCs. These observations suggest that the RML scrapie isolate, unlike the ME7 scrapie isolate, may utilize both FDCs and B lymphocytes, but the exact roles of these two cell types are not clear. However, direct comparisons of the peripheral pathogenesis of the RML scrapie isolate and the ME7 scrapie strain are required to demonstrate that the discrepancy between them is not due to different experimental practices.

The apparent differences in the pathogenesis of the ME7 and RML scrapie isolates have important implications, as they suggest that different scrapie strains may target different cell populations in lymphoid tissues. Current evidence suggests a similar variation in human TSE diseases, as PrP^{Sc} is detected in non-CNS tissues, including lymph nodes, tonsils (18), and appendix (19), from patients with vCJD but not from patients with sporadic or even iatrogenic CJD, where infection is introduced via the periphery. The risk of horizontal infection through transfusion and transplantation of vCJD-contami-

nated tissue or use of contaminated surgical instruments is a matter of urgent concern, particularly given the fact that intraspecies transmissions usually increase the risk of infection.

Once TSEs spread to the CNS, the neurodegeneration they cause is most likely irreversible. Treatments that interfere with the early stages of infection in peripheral tissues can significantly decrease scrapie susceptibility (8, 9). Therefore, the identification of FDCs as critical cells in the peripheral pathogenesis of TSE diseases is fundamental for determining the risk of iatrogenic spread and the development of practical prophylactic and therapeutic strategies.

ACKNOWLEDGMENTS

We thank Jean Langhorne (Imperial College, London, United Kingdom) for providing the $\text{IL-6}^{-/-}$ mice, Marie Kosco-Vilbois (Sero Pharmaceutical Research Institute, Geneva, Switzerland) for helpful discussion and provision of FDC-M1 monoclonal antiserum, and Louise Gibbard (Institute for Animal Health, Compton, United Kingdom), Irene McConnell, Mary Brady, and Jenny Beaton (Institute for Animal Health, Neuropathogenesis Unit, Edinburgh, United Kingdom) for excellent technical support.

This work was supported by funding from the Medical Research Council and the Biotechnology and Biological Sciences Research Council.

REFERENCES

- Beekes, M., P. A. McBride, and E. Baldauf. 1998. Cerebral targeting indicates vagal spread of infection in hamsters fed with scrapie. *J. Gen. Virol.* **79**:601–607.
- Bellinger, D. L., S. Y. Felten, D. Lorton, and D. L. Felten. 1997. Innervation of lymphoid organs and neurotransmitter-lymphocyte interactions, p. 226–329. In R. W. Keane and W. F. Hickey (ed.), *Immunology of the nervous system*. Oxford University Press, Oxford, United Kingdom.
- Blättler, T., S. Brander, A. J. Raeber, M. A. Klein, T. Voigtländer, C. Weissmann, and A. Aguzzi. 1997. PrP-expressing tissue required for transfer of scrapie infectivity from spleen to brain. *Nature* **389**:69–73.
- Bolton, D. C., M. P. McKinley, and S. B. Prusiner. 1982. Identification of a protein that purifies with the scrapie prion. *Science* **218**:1309.
- Brown, K. L., K. Stewart, D. Ritchie, N. A. Mabbott, A. Williams, H. Fraser, W. I. Morrison, and M. E. Bruce. 1999. Scrapie replication in lymphoid tissues depends on PrP-expressing follicular dendritic cells. *Nat. Med.* **5**:1308–1312.
- Bruce, M. E., R. G. Will, J. W. Ironside, I. McConnell, D. Drummond, A. Suttie, L. McCardle, A. Chree, J. Hope, C. Birkett, S. Cousens, H. Fraser, and C. J. Bostock. 1997. Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. *Nature* **389**:498–501.
- Büeler, H., M. Fischer, Y. Lang, H. Bluethmann, H.-P. Lipp, S. J. DeArmond, S. B. Prusiner, M. Aguet, and C. Weissmann. 1992. Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein. *Nature* **356**:577–582.
- Farquhar, C., A. Dickinson, and M. Bruce. 1999. Prophylactic potential of pentosan polysulphate in transmissible spongiform encephalopathies. *Lancet* **353**:117.
- Farquhar, C. F., and A. G. Dickinson. 1986. Prolongation of scrapie incubation period by an injection of dextran sulphate 500 within the month before or after infection. *J. Gen. Virol.* **67**:463–473.
- Farquhar, C. F., J. Dornan, R. A. Somerville, A. M. Tunstall, and J. Hope. 1994. Effect of *Sinc* genotype, agent isolate and route of infection on the accumulation of protease-resistant PrP in non-central nervous system tissues during the development of murine scrapie. *J. Gen. Virol.* **75**:495–504.
- Farquhar, C. F., R. A. Somerville, and M. E. Bruce. 1998. Straining the prion hypothesis. *Nature* **391**:345–346.
- Farquhar, C. F., R. A. Somerville, and L. A. Ritchie. 1989. Post-mortem immunodiagnosis of scrapie and bovine spongiform encephalopathy. *J. Virol. Methods* **24**:215–222.
- Fraser, H., K. L. Brown, K. Stewart, I. McConnell, P. McBride, and A. Williams. 1996. Replication of scrapie in spleens of SCID mice follows reconstitution with wild-type mouse bone marrow. *J. Gen. Virol.* **77**:1935–1940.
- Fraser, H., and A. G. Dickinson. 1973. Agent-strain differences in the distribution and intensity of grey matter vacuolation. *J. Comp. Pathol.* **83**:29–40.
- Fraser, H., and A. G. Dickinson. 1978. Studies on the lymphoreticular system in the pathogenesis of scrapie: the role of spleen and thymus. *J. Comp. Pathol.* **88**:563–573.
- Fraser, H., and C. F. Farquhar. 1987. Ionising radiation has no influence on scrapie incubation period in mice. *Vet. Microbiol.* **13**:211–223.
- Grau, G. E., P.-F. Piguet, P. Vassalli, and P.-H. Lambert. 1989. Tumour necrosis factor and other cytokines in cerebral malaria. *Immunol. Rev.* **112**:49–70.
- Hill, A. F., M. Zeider, J. Ironside, and J. Collinge. 1997. Diagnosis of new variant Creutzfeldt-Jakob disease by tonsil biopsy. *Lancet* **349**:99–100.
- Hilton, D., E. Fathers, P. Edwards, J. Ironside, and J. Zajicek. 1998. Prion immunoreactivity in appendix before clinical onset of variant Creutzfeldt-Jakob disease. *Lancet* **352**:703–704.
- Kapasi, Z. F., G. F. Burton, L. D. Schultz, J. G. Tew, and A. K. Szakal. 1993. Induction of functional follicular dendritic cell development in severe combined immunodeficiency mice. *J. Immunol.* **150**:2648–2658.
- Kitamoto, T., T. Muramoto, S. Mohri, K. Doh-Ura, and J. Tateishi. 1991. Abnormal isoform of prion protein accumulates in follicular dendritic cells in mice with Creutzfeldt-Jakob disease. *J. Virol.* **65**:6292–6295.
- Klein, M. A., R. Frigg, E. Fleischig, A. J. Raeber, U. Kalinke, H. Bluethmann, F. Bootz, M. Suter, R. M. Zinkernagel, and A. Aguzzi. 1997. A crucial role for B cells in neuroinvasive scrapie. *Nature* **390**:687–691.
- Klein, M. A., R. Frigg, A. J. Raeber, E. Fleischig, I. Hegyi, R. M. Zinkernagel, C. Weissmann, and A. Aguzzi. 1998. PrP expression in B lymphocytes is not required for prion neuroinvasion. *Nat. Med.* **4**:1429–1433.
- Klinkert, W. E. F., K. Kojima, W. Lesslauer, W. Rinner, H. Lassmann, and H. Wekerle. 1997. TNF- α receptor fusion protein prevents experimental auto-immune encephalomyelitis and demyelination in Lewis rats: an overview. *J. Neuroimmunol.* **72**:163–168.
- Kopf, M., H. Baumann, G. Freer, M. Freudenberg, M. Lamers, T. Kishimoto, R. Zinkernagel, H. Bluethmann, and G. Kohler. 1994. Impaired immune and acute-phase responses in interleukin-6-deficient mice. *Nature* **368**:339–342.
- Kopf, M., S. Herren, M. V. Wiles, M. B. Pepys, and M. H. Kosco-Vilbois. 1998. Interleukin 6 influences germinal center development and antibody production via a contribution of C3 complement component. *J. Exp. Med.* **188**:1895–1906.
- Kordek, R., V. R. Nerurkar, P. L. Liberski, S. Isaacson, R. Yanagihara, and D. C. Gadjusek. 1996. Heightened expression of tumor necrosis factor α , interleukin 1 α , and glial fibrillary acidic protein in experimental Creutzfeldt-Jakob disease in mice. *Proc. Natl. Acad. Sci. USA* **93**:9754–9758.
- Kosco-Vilbois, M. H., H. Zentgraf, J. Gerdes, and J.-Y. Bonnefoy. 1997. To "B" or not to "B" a germinal center? *Immunol. Today* **18**:225–230.
- Le Hir, M., H. Bluethmann, M. H. Kosco-Vilbois, M. Muller, F. di Padova, M. Moore, B. Ryffel, and H.-P. Eugster. 1996. Differentiation of follicular dendritic cells and full antibody responses require tumour necrosis factor receptor-1 signalling. *J. Exp. Med.* **183**:2367–2372.
- Leist, T. P., K. Frei, S. Kam-Hansen, R. M. Zinkernagel, and A. Fontana. 1988. Tumour necrosis factor in cerebrospinal fluid during bacterial, but not viral, meningitis. *J. Exp. Med.* **167**:1743–1748.
- Mabbott, N. A., K. L. Brown, J. Manson, and M. E. Bruce. 1997. T lymphocyte activation and the cellular form of the prion protein. *Immunology* **92**:161–165.
- Mackay, F., and J. L. Browning. 1998. Turning off follicular dendritic cells. *Nature* **395**:26–27.
- Matsumoto, M., S. Mariathasan, M. H. Nahm, F. Baranay, J. J. Peschon, and D. P. Chaplin. 1996. Role of lymphotoxin and the type 1 TNF receptor in the formation of germinal centers. *Science* **271**:1289–1291.
- McBride, P., P. Eikelenboom, G. Kraal, H. Fraser, and M. E. Bruce. 1992. PrP protein is associated with follicular dendritic cells of spleens and lymph nodes in uninfected and scrapie-infected mice. *J. Pathol.* **168**:413–418.
- McBride, P. A., and M. Beekes. 1999. Pathological PrP is abundant in sympathetic and sensory ganglia of hamsters fed with scrapie. *Neurosci. Lett.* **265**:135–138.
- Meda, L., M. A. Cassatella, G. I. Szendrei, L. Otvos, P. Baron, M. Villalba, D. Ferrari, and F. Rossi. 1995. Activation of microglial cells by β -amyloid protein and interferon- γ . *Nature* **374**:647–650.
- Meyer, R. K., M. P. McKinley, K. A. Bowman, M. B. Braunfeld, R. A. Barry, and S. B. Prusiner. 1986. Separation and properties of cellular and scrapie prion proteins. *Proc. Natl. Acad. Sci. USA* **83**:2310–2314.
- Pasparakis, M., L. Alexopoulou, V. Episkopou, and G. Kollias. 1996. Immune and inflammatory responses in TNF α -deficient mice: a critical requirement for TNF α in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centres, and in the maturation of the humoral immune response. *J. Exp. Med.* **184**:1397–1411.
- Probert, L., K. Akassoglou, G. Kassiotis, M. Pasparakis, L. Alexopoulou, and G. Kollias. 1997. TNF- α transgenic and knockout models of CNS inflammation and degeneration. *J. Neuroimmunol.* **72**:137–141.
- Prusiner, S. B., D. C. Bolton, D. F. Groth, K. A. Bowman, S. P. Cochran, and M. P. McKinley. 1982. Further purification and characterisation of scrapie prions. *Biochemistry* **21**:6942–6950.
- Raeber, A. J., M. A. Klein, R. Frigg, E. Fleischig, A. Aguzzi, and C. Weissmann. 1999. PrP-dependent association of prions with splenic but not circulating lymphocytes of scrapie-infected mice. *EMBO J.* **18**:2702–2706.
- Raine, C. S. 1995. Multiple sclerosis: TNF revisited, with promise. *Nat. Med.* **1**:211–214.
- Ritchie, D. L., K. L. Brown, and M. E. Bruce. 1999. Visualisation of PrP protein and follicular dendritic cells in uninfected and scrapie infected spleen. *J. Cell. Pathol.* **4**:3–10.
- Taraboulos, A. T., K. Jendroska, D. Serban, S.-L. Yang, S. J. DeArmond, and S. B. Prusiner. 1992. Regional mapping of prion proteins in brain. *Proc. Natl. Acad. Sci. USA* **89**:7620–7624.
- Taylor, D. M., I. McConnell, and H. Fraser. 1996. Scrapie infection can be established readily through skin scarification in immunocompetent but not immunodeficient mice. *J. Gen. Virol.* **77**:1595–1599.
- Tkachuk, M., S. Bolliger, B. Ryffel, G. Pluschke, T. A. Banks, S. Herren, R. H. Gisler, and M. H. Kosco-Vilbois. 1998. Crucial role of tumour necrosis factor receptor 1 expression on nonhematopoietic cells for B cell localization within the splenic white pulp. *J. Exp. Med.* **187**:469–477.
- Williams, A., A.-M. van Dam, D. Ritchie, P. Eikelenboom, and H. Fraser. 1997. Immunocytochemical appearance of cytokines, prostaglandin E2 and lipocortin-1 in the CNS during the incubation period of murine scrapie correlates with progressive PrP accumulations. *Brain Res.* **754**:171–180.
- Wu, Q., Y. Wang, J. Wang, E. O. Hedgeman, J. L. Browning, and Y.-X. Fu. 1999. The requirement of membrane lymphotoxin for the presence of dendritic cells in lymphoid tissues. *J. Exp. Med.* **190**:629–638.